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REVIEW
Freeze-drying of proteins: some emerging concerns

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Freeze-drying (lyophilization) removes water from a frozen sample by sublimation and desorption. It can be viewed as a three-step process consisting of freezing, primary drying and secondary drying. While cryoprotectants can protect the protein from denaturation during early stages, lyoprotectants are needed to prevent protein inactivation during drying. The structural changes as a result of freeze-drying have been investigated, especially by FTIR (Fourier-transform IR) spectroscopy. In general, drying results in a decrease of α-helix and random structure and an increase in β-sheet structure. In the case of basic fibroblast growth factor and γ-interferon, enhanced FTIR showed large conformational changes and aggregation during freeze-drying, which could be prevented by using sucrose as a lyoprotectant. It is now well established that structural changes during freeze-drying are responsible for low activity of freeze-dried powders in nearly anhydrous media. Strategies such as salt activation can give 'activated' enzyme powders, e.g. salt-activated thermolysin-catalysed regioselective acylation of taxol to give a more soluble derivative for therapeutic use. In the presence of moisture, freeze-dried proteins can undergo disulphide interchange and other reactions which lead to inactivation. Such molecular changes during storage have been described for human insulin, tetanus toxoid and interleukin-2. Some successful preventive strategies in these cases have also been mentioned as illustrations. Finally, it is emphasized that freeze-drying is not an innocuous process and needs to be understood and used carefully.

Introduction

Freeze-drying (lyophilization) is perceived to be a gentle process for concentrating or drying biologically active substances. Enzymologists have been using it as a routine technique for more than four decades, and pharmaceutical industries also adopted it quite some time ago. This is because enzyme/protein- or peptide-based drugs are more stable in solid (as compared with solution) form. Thus freeze-dried powders offer advantages at the storage and shipping/distribution stages. They also increase the shelf-life at the end-user stage [1]. The two reasons why a greater understanding of freeze-drying has assumed an urgent and considerable relevance to life science and pharmaceutical science are as follows: (1) it has been realized in recent years that stability of freeze-dried powders depends critically on how the freeze-drying is carried out [2,3]; this is of great concern to producers of enzymes and other biologically active proteins; (2) in the last few years it has been realized that the low activity of freeze-dried enzyme powders in nearly anhydrous organic solvents is largely due to protein inactivation at the stage of freeze-drying; non-aqueous enzymology is employed for many biotechnologically important bioconversions, synthesis and resolution of stereoisomers [4–6].

In view of these factors, there is a need to evolve techniques to minimize this form of inactivation and improve catalytic efficiency during the target process. This, of course, involves basic understanding of freeze-drying, which is a complex process.

Basic process [7]

Freeze-drying removes water from a frozen sample by sublimation and desorption. All freeze-driers (lyophilizers), whether a simple glass unit fabricated in a glass-blowing laboratory or a sophisticated industrial-scale unit, have two essential design features: a low-pressure chamber to which the frozen sample is attached and a 'cold finger' or 'cold trap' which collects the sublimated or desorbed ice. The process of freeze-drying can be visualized in terms of three steps [2]. These three stages have been discussed in detail fairly recently [8], so it may thus suffice here to present a brief qualitative picture only. It may,

Key words: cryoprotectants, freeze-drying, lyoprotectants, protein aggregation, protein lyophilization, storage stability of pharmaceuticals.

Abbreviations used: FTIR, Fourier-transform IR; PMR, proton magnetic resonance; T_g, glass transition temperature of the dried product; T_g', glass transition temperature associated with maximum freeze concentration; T_{mc}, critical temperature of molecular mobility; W_g', amount of frozen water at glass transition temperature; pCl, $-\log [Cl^-]$.

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however, be stressed that process validation requires strict control of various parameters at all the three stages during various cycles (http://www.fda.gov/ora/inspect_ref/igs/lyophi.html).

(a) Initial freezing

Freezing involves formation of ice nuclei. Depending upon many factors, especially the cooling rate, supercooling may occur. Thus ice formation may, in fact, take place well below 0 °C [8]. Faster freezing rates give rise to small ice crystals. The result is faster sublimation at the primary drying phase, but slower secondary drying. Removal of water by freezing increases the solute concentration and hence the viscosity. At some stage, a saturation value is reached and no further increase in concentration/viscosity is possible. At this stage, the glass transition [involving the parameter T_g' (glass transition temperature associated with maximum freeze concentration)] takes place. "A glass is a supersaturated, thermodynamically unstable liquid with the viscosity as high as 10^{12-14} Pa · s" [9]. In other words, "a glass transition is not a true thermodynamic change in state, but instead is a kinetic limit at high viscosity" [10]. Two other parameters that are important, are W_g' (the unfrozen water at this temperature) and T_g (the glass transition temperature of the dried product) [11]. Both W_g' and T_g can be measured by differential scanning calorimetry [11].

(b) Primary drying

This is the stage at which ice separated from the solute phase is removed by sublimation. If the sample is thick, mass transfer constraints decrease the rate of sublimation [8]. The temperature of the sample is a rather critical parameter; if the temperature rises above T_g', the ice would melt into the solute phase. At the same time, a temperature rise of 1 °C is reported to make drying faster by 13% [8]. Hatley [11] has shown that carrying out primary drying above T_g' produces freeze-dried samples of inferior quality.

(c) Secondary drying

This stage begins after all the frozen water has sublimed and thus can be facilitated by increasing the product temperature. In the case of proteins, this temperature has to be chosen while keeping in view the thermal stability of proteins. The 'bound water' (to protein molecules) or the water molecules 'trapped' in the glass phase are removed during this stage. Up to the 2% level, the water is removed quickly, and this process slows down thereafter. This is because, as the sample dries, diffusion of water molecules through the sample becomes more difficult. It is a function of the porosity of the sample and does not depend much upon the sample thickness [8].

In the case of food proteins, there is extensive data to indicate that storage temperature of the freeze-dried proteins should be kept higher than their T_g. This is necessary to prevent collapse. The volumetric shrinkage after collapse, the importance of sample mass, etc., have also been discussed. There are sufficient data to suggest that similar factors are important in the case of freeze-dried proteins [8]. Excipients, which result in higher T_g or higher collapse temperature, result in greater stability of freeze-dried proteins and vice versa. It is also known that excipients that facilitate phase separation during freeze-drying, accelerate protein inactivation [12].

The presence of excipients during freeze-drying obviously leads to a more complicated phase diagram at the freezing and primary drying stages [13]. During the subsequent discussion of the effect of excipients, the explanations have relied on the more widely accepted theory of 'water substitution' [8]. However, it may be worth mentioning straight away that the 'vitrification hypothesis' presents another viewpoint. According to some workers, proteins in glassy state exhibit decreased molecular motion, which slows down many degradative processes [8,9]. However, as Craig et al. [8] have pointed out, a clear link between T_g of the formulation and storage stability of the product has not been established.

Bound water and protein activity

The classical experiments by Rupley et al. [14] indicated the importance of a minimum amount of water in making the enzyme molecule catalytically active. Using heat capacity and IR spectroscopy, Rupley et al. [14] showed that water molecules were deposited on dry lysozyme and the latter became biologically active even before the monolayer of water molecules around the enzyme molecule had formed. Subsequent work indicated that water 'deposition' follows a definite sequence. First it gets deposited on charged and polar amino acids, and, next, around the hydrophobic clusters. The former stage is vital, since, in the absence of water molecules, the side chains of amino acids interact with each other and 'lock up' the conformation. This robs the enzyme molecule of the flexibility that is necessary for its catalytic activity [15,16]. This rigidity, in the absence of enough water, is in fact responsible for many enzyme molecules being stable in the dry state even at 100 °C in anhydrous organic solvents [17], even though the same enzyme may get completely inactivated at much lower temperatures when heated in its aqueous solution.

Freeze-drying and protein stability

There are two kinds of protein stability which are of importance. First is the stability during storage and transport of the protein. This is important to both enzyme

manufacturers, who have to ship the biologically active proteins to distant places, and to the life scientist, who has to decide storage conditions and permissible shelf-life. It is of special concern in the case of pharmaceutically important proteins and vaccines in the context of healthcare in developing countries where a 'cold chain' is not necessarily available for storage. The second kind of stability is operational stability, which describes how stable the enzyme is during catalysis. This stability can often be higher, since the presence of the substrate may enhance the conformational stability of the enzyme [18]. The operational stability can also be drastically reduced if the components of the 'substrate preparation' include a molecule which inactivates the enzyme. A good illustration of this is the reduced operational stability of lactase during hydrolysis of whey lactose [19]. In the following discussion, we are mostly concerned with storage stability.

It has been known for a long time that most freeze-dried RNase A samples contain dimers and aggregates [20]. The amount of these enzymically active aggregates increases significantly if the freeze-drying is carried out from 50% acetic acid. An interesting result worth recalling is that a dimer formed from inactive alkylated monomers modified at His^{12} and His^{119} had 50% activity, since these modified monomer molecules fashioned an active site consisting of unmodified His^{12} and His^{119} [21]. The second important, even if obvious, comment is that the mechanisms of protein inactivation are not different, irrespective of which stress factor is applied. Proteins at high temperatures in aqueous buffers or in anhydrous organic solvents or at high pressure, follow a similar mechanistic route (at the molecular level) during their inactivation. Protein inactivation can be either reversible or irreversible [22]. During the reversible phase, the protein chain unfolds and can go back to the native structure if the 'denaturing factor' is removed. Exposure to moderately high temperatures such as 40–45 °C for a few minutes or 8 M urea are two examples of such denaturing conditions. Prolonged heat treatment at higher temperatures will cause irreversible changes in the protein molecule. Molecular mechanisms for these irreversible changes are fairly well understood and have been briefly discussed [23]. Apart from conformational changes (which may be reversible at early stages), proteins eventually undergo a number of other structural changes [13]. Unfolding of protein chains exposes many hydrophobic residues which were buried in the native structure. Intermolecular hydrophobic interaction between such residues generally leads to protein aggregation. The disulphide exchange reaction, another inactivation mechanism, causes mispairing of thiol groups, either of intermolecular or intramolecular nature. Inactivation mechanisms also include some chemical changes which lead to irreversible loss of activity in solution or during the post-freeze-drying stage. Such changes can also occur, especially if extreme pH conditions prevail during freeze-drying. Cases of hydrolysis of peptide bonds (next to aspartic acid residues), deamidation of asparagine residues and glutamine, and racemization of amino acid residues have been well-documented [24]. In the case of glycoproteins or if free reducing sugars are present in the sample, the Maillard reaction plays an important role in determining the overall stability [24]. Many of these reactions can be minimized by ensuring that the protein is not exposed to high temperatures at any stage. The relative role played by a particular chemical change may depend considerably upon the stress factor or the particular protein. The third point, which is also worth remembering, is that (although the same or similar inactivation mechanisms operate), structural changes during freeze-drying itself and the post-freeze-drying stage (storage) need to be identified. This distinction is often overlooked. This happens because, for a variety of end applications, one tends to look at the activity at the time of usage. For example, in the pharmaceutical industry, all operations beyond purification stage, such as freezing, thawing, formulation, sterile filtration, filling, freeze-drying and inspection are called 'formulation' or 'fill-finish operations' [25]. The whole issue is complicated further by the fact that reconstitution conditions may decide the ultimate recovery. Webb et al. [26], while working with recombinant human interferon-γ, showed "the potential for recovery of native protein using the appropriate reconstitution conditions, even though the protein is non-native in the lyophilized state".

Protein stability in solution [23,27]

During early stages of freeze-drying, the protein is in fact 'in solution'. Partial unfolding, which happens during this interfacial phenomenon, is largely reversible. Millqvist-Fureby et al. [28] have described the use of electron spectroscopy for chemical analysis to show the existence of surface of freeze-dried solid enriched in protein. Earlier, Eckhardt et al. [29] showed that rapid freezing promotes aggregation of human growth hormone. This is in agreement with the observation of Hsu et al. [30] that the rate of development of turbidity in reconstituted freeze-dried solids increased with the increased surface areas of the solids. In fact, Sarciaux et al. [31] have shown that annealing reduces the specific surface area of freeze-dried solids and aggregation of bovine IgG.

Protein stability during freeze-thawing

Arakawa et al. [2] have provided a comprehensive treatment of factors affecting protein stability during freeze-drying. Their review makes a distinction between the

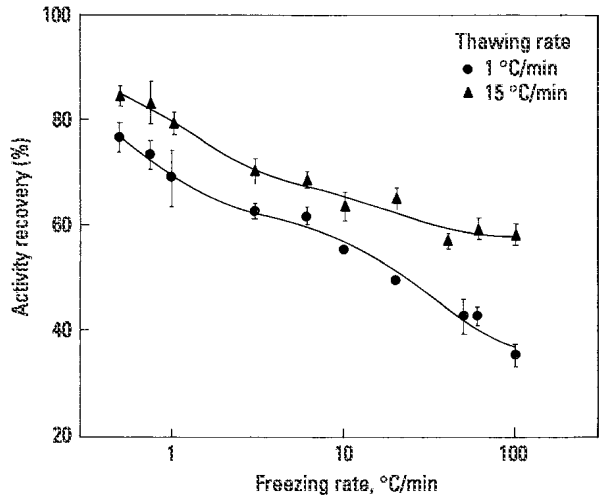

Figure 1 Effect of freezing rate on recovery of activity of lactate dehydrogenase

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freeze–thawing and freeze-drying processes in the context of protein stability. Figure 1 shows the importance of freezing and thawing rates on the recovery of enzyme activity. The maximum recovery of activity occurred with slow freezing and fast thawing. It is reported that slow freezing leads to large ice crystals, whereas fast freezing leads to fine ice crystals [32]. The latter increased the interfacial area considerably and led to denaturation. Figure 2 shows that freezing damage increased with the addition of salt throughout the range of freezing rates. This is attributed to the formation of Na_2HPO_4 from K_3PO_4 and NaCl; Na_2HPO_4 precipitates at low temperature and reduces the pH of the unfrozen buffer. Arakawa et al. [2] have presented experimental evidence to show that a co-solute has the same stabilization or destabilization effect during freeze–thawing as it has during the solution stage. However, the co-solutes behave differently during freeze-drying, which led Crowe et al. [33] to conclude that mechanisms of destabilization during freeze-drying are different from freeze–thawing. The osmolytes protect proteins during freeze–thawing and the preferential exclusion theory developed by Timasheff and co-workers [34,35] can be extended to freeze–thawing. According to the preferential exclusion theory, the solutes are excluded from the vicinity of the protein and the protein is preferentially hydrated. Numerous compounds ('cryoprotectants'), with widely differing chemical structures, such as sugars, amino acids, amines, polyols and some salts, if present with the protein, act as protectors during freeze–thawing. Compounds such as $MgCl_2$, guanidinium chloride, KSCN and urea show weak preferential exclusion or preferential binding and destabilize

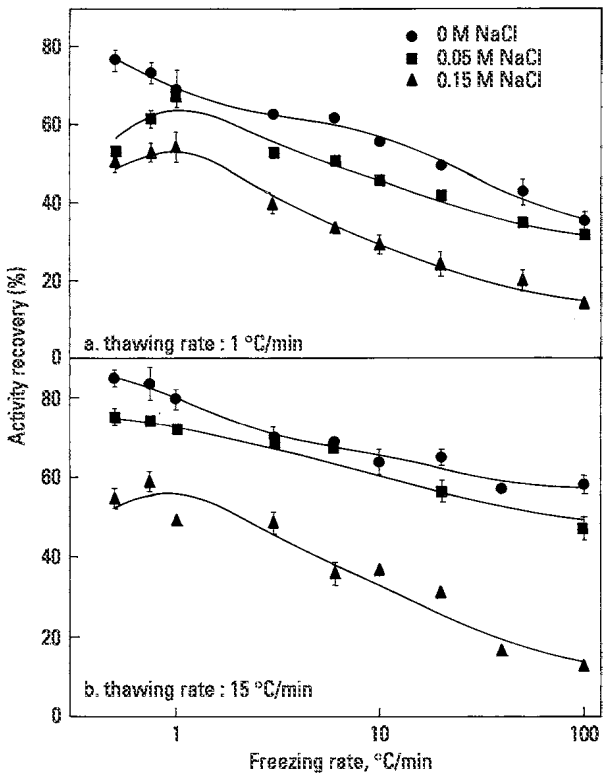

Figure 2 Effect of freezing rate on the recoveries of activities of (a) lactate dehydrogenase and (b) alcohol dehydrogenase in the presence of NaCl

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the protein in solution, and thus are expected to play the same role during freeze–thawing. Similarly, pH, protein concentration, and the presence of cofactors and allosteric modifiers, which favour protein stability in solution, will also stabilize a protein during freeze–thawing. This is not unexpected, since during freezing, protein molecules are present in the non-ice phase along with the co-solute. The concentration of the co-solute is also very high, since bulk water has formed ice.

Lyoprotectants: protein stability during freeze-drying

Freeze-drying involves two denaturing conditions: freezing and drying. Stabilization of proteins during freeze-drying can be attempted by adding excipients to the protein solution. In order to be effective, these excipients have to protect the protein structure during both 'stress factors' of freezing and drying. As pointed out by Arakawa et al. [2], "Many effective cryoprotectants have no stabilizing effect during dehydration. The only solutes that do provide stabilization are disaccharides. This mechanism is not yet

fully understood but it can not involve preferential exclusion of co-solutes". Carpenter and Crowe [36,37] have used FTIR (Fourier-transform IR) spectroscopy to show that certain carbohydrates protect proteins by H-bonding, a role fulfilled by water molecules which are taken away during drying. However, in the case of lysozyme, FTIR spectroscopy showed that the presence of trehalose during freeze-drying did not prevent the structural changes completely [38]. The work of Lippert and Galinski [39] with phosphofructokinase and lactate dehydrogenase also showed H-bonding to be responsible for stabilization during the drying process.

Prestrelski et al. [40] used resolution-enhanced FTIR to show that basic fibroblast growth factor, interferon-γ and α-lactalbumin underwent large conformational changes and aggregation during freeze-drying, and demonstrated that these changes could be prevented by the presence of sucrose. Other carbohydrates such as glucose fail to do so, since they fail to act as cryoprotectants during freezing. The concentration at which glucose could have worked as a cryoprotectant leads to crystallization of the sugar during freeze-drying, so that the molecule is no more available for H-bonding with the protein. Carpenter et al. [41] and Prestrelski et al. [42] have suggested a two-component excipient system in which poly(ethylene glycol) acts as a cryoprotectant and carbohydrates act as lyoprotectants. In such systems, glucose at low concentrations does act as a lyoprotectant. Prestrelski et al. [42] have also shown that, unless the native structure is preserved during the drying process (with the help of excipients), rehydration does not lead to the recovery of active proteins. However, later work by Webb et al. [26] raises the possibility of activity recovery from non-active structure at the reconstitution stage.

Strambini and Gabellieri [43] tracked the amide I signal (by FTIR) and the secondary structure after rehydration (monitored by CD spectroscopy) and biological activity of actin (measured by polymerization assay). It was found that: (i) there was loss of secondary structure during freeze-drying; (ii) "structural perturbations induced by dehydration are still retained, up to some degree, upon rehydration"; (iii) "unfolding induced by freeze-drying actin with 1% sucrose appeared to be fully reversible upon rehydration. The CD structure of the rehydrated sample is almost identical to that of native actin"; (iv) however, the level of recovered activity (33%) in the above case was low. Thus monitoring of secondary structure in the solid state and rehydrated samples could be misleading if the data are directly interpreted in terms of recovery of biological activity. The authors observe, "Protein activity depends upon higher order structural organization in addition to secondary structure". Another important observation made by these authors is that the presence of co-solutes at the rehydration stage may also promote refolding over aggregation. Carrasquillo et al. [44] found no correlation between the increased conformational stability in aqueous solution and the freeze-drying-induced structural changes in α-chymotrypsin. This is contradictory to the earlier observation of Arakawa et al. [2] that, "in general, any factor that alters protein stability in non-frozen aqueous solution will tend to have the same qualitative effect during freeze-thawing". A useful observation made is that β-sheet proteins seem to resist such changes better than α-helical proteins.

Another practical observation is by Jiang and Nail [45] who worked with catalase, β-galactosidase and lactate dehydrogenase, and concluded that the most important drying process variable affecting recovery of biological activity was residual moisture level, with a considerable decrease in recoverable activity being associated with moisture levels below 10%.

The freeze-drying of multimeric proteins has also been investigated. In addition to the prevention of unfolding, preferentially excluded solids also promote quaternary-structure formation. Thus, in the case of lactate dehydrogenase, maintenance of the tetrameric form in the frozen state during primary drying was found to be critical for enzyme activity after freeze-drying, and thus BSA and polyvinylpyrrolidone were found to be useful additives [46].

Yoshioka et al. [47] have examined the NMR-relaxation-based critical temperature of molecular mobility (T_{mc}) and T_g (by differential scanning calorimetry) of freeze-dried γ-globulin formulations containing different polymer excipients. The molecular mobility of water in the formulations was measured by PMR (proton magnetic resonance) and dielectric relaxation spectrometry. Not surprisingly, T_{mc} was found to increase as the extent of bound water increased. As T_{mc} reflected the stability of formulations, dextran was found to be a better additive as compared with methylcellulose.

Freeze-dried enzyme powders in nearly anhydrous media

Most enzymology is concerned with reactions of enzymes in aqueous buffers. Even in the *in vivo* context, aqueous medium is the one that is relevant for discussing the biological activity of enzymes and proteins. However, use of enzymes in non-aqueous media offers numerous advantages (Table 1), especially in the synthesis of fine chemicals (including drug intermediates). Freeze-dried enzymes have often been used in such applications [5]. Thus it is useful to discuss the behaviour of freeze-dried enzyme powders in such media. It was in the late 1960s that Price and co-workers used chymotrypsin and xanthine oxidase in dry organic solvents and found these enzyme powders to be catalytically active [48–50]. However, it was in the 1980s

Table 1 Advantages of using enzymes in non-aqueous media [4,5]

- Hydrolases can be used for synthesis
- Greater storage stability
- No contamination, because microbial growth is absent
- A large number of media are possible, with a wide range of polarity and hydrophobicity
- Possible range of reaction temperature is expanded
- Increased repertoire of reactions, in terms of regiospecificity, stereospecificity, involvement of compounds soluble in organic media, etc.
- Biotransformations by whole cells are also possible
- It is possible to control stereoselectivity by varying temperature and water activity
- Separation of reactants and products from the enzyme is not a problem, since the enzymes are practically insoluble in organic solvents

that modern phase of non-aqueous enzymology started, with some pioneering work from various groups [51–55]. It was shown that: (i) freeze-dried powders of several enzymes, such as α-chymotrypsin, subtilisin, lipases and tyrosinase are active in various organic solvents [51,53,54]. Such media have been described as nearly anhydrous, low-water-containing [56] or neat organic solvents [57]; (ii) enzyme powders gave maximum activity when freeze-dried from an aqueous buffer with a pH equivalent to the optimum pH of the enzyme. This process was called 'pH tuning' and the phenomenon called 'pH memory' ("it was as if the enzyme remembered the pH of the solution from which it had been lyophilized" [58]). This phenomenon will be described in greater detail later on; (iii) in general, more hydrophobic solvents, when used as reaction media, gave higher catalytic rates as compared with hydrophilic solvents. For example, subtilisin showed a V_{max}/K_m ratio of 2×10^{-3} min^{-1} in n-octane as compared with $< 1 \times 10^{-7}$ min^{-1} in DMSO [53]. This is sometimes a disadvantage, since many substrates have low solubility in hydrophobic solvents. Thus, when hydrophilic solvents such as DMSO are used, the reaction rates for the processes are quite low. Several parameters of the medium have been suggested to correlate the activity of freeze-dried enzyme powders with nature of the medium [59,60]. None has been found to be totally satisfactory, but log P (defined as the partition coefficient of the organic solvent in n-octanol to water), which was one of the earliest proposed parameters, has been generally considered to be the most acceptable [61]. Solvents with log $P < 2$ are poor, those with log $P > 4$ are the most suitable and the solvents in the intermediate range (between 2 and 4) are unpredictable [61]. The general model, which is accepted in this regard, is that the more hydrophilic solvents strip off the essential water layer from the enzyme and render the protein molecules inactive [53,62]. This happens because, in the absence of water, the side chains of amino acids start interacting with each other and the conformation loses the necessary flexibility for catalysis.

An interesting development has been that the essential water layer can be replaced by polar solvents [63]. The solvents having log P values between 1.08 and 1.93 have been recommended for this purpose [64].

Low catalytic activity of freeze-dried enzyme powders

Klibanov [65] and Lee and Dordick [66] have discussed the reasons responsible for the low activity of freeze-dried enzyme powders in organic solvents. Diffusional limitations, unfavourable energetics of substrate desolvation, transition-state destabilization and conformational changes during freeze-drying all contribute to various extents. A clear quantitative picture about their relative roles with an adequate number of enzymes is not available. In fact, words like 'dramatic', etc., so frequently used in non-aqueous enzymology, have succeeded in taking attention away from the fact that enzyme powders do have low catalytic power in organic solvents as compared with aqueous buffers. There are only limited data on the relative catalytic efficiencies in the two different media. The k_{cat} values for esterase and transesterification activities of subtilisin (with hexane as the reaction medium) are 5.9×10^3 and 1.04×10^{-1} $M^{-1} \cdot s^{-1}$ respectively [66]. Two lines of investigation, namely spectroscopic studies (mostly FTIR spectroscopy) of freeze-dried enzyme powders and partially successful attempts at preventing loss of enzyme activity during freeze-drying, indicate that freeze-drying is the key factor responsible for the low activity of enzyme powders in organic media.

'Activated' freeze-dried enzymes for use in nearly anhydrous media

Lee and Dordick [66] have recently provided an excellent list of strategies which yield freeze-dried enzyme powders showing high activity in nearly anhydrous media. In most of these strategies, the common feature is to use an additive which prevents drastic structural changes in the enzyme during 'drying'. An important point made by the authors is "... because enzyme activity and selectivity are kinetically equivalent (through the catalytic efficiency term, V_{max}/K_m), many methods discovered in recent years have also led to tailored enzyme selectivity" [66]. Table 2 lists some examples of enzymes that have exhibited the phenomenon of 'activation' in organic solvents.

pH-tuned freeze-dried enzymes

pH tuning is necessary in many cases to obtain significant activity in non-aqueous media. Later work, especially by Halling and co-workers, has given fairly good understanding

Table 2 List of additives used to activate enzymes in non-aqueous media

Enzyme	Solvent	Additive	Activation factor	Reference(s)
Protease (*Aspergillus oryzae*)	Anhydrous pyridine	Sorbitol	64	[116]
		Sucrose	38	[116]
		Xylitol	32	[116]
		Trehalose	26	[116]
		Mannitol	20	[116]
		Poly(ethylene glycol)	8	[116]
α-Chymotrypsin	Anhydrous pyridine	Sorbitol	19	[116]
Subtilisin Carlsberg	Anhydrous pyridine	Sorbitol	45	[116]
	Anhydrous 2-methylbutan-2-ol[a]	17-β-Oestradiol	10	[82]
	Hexane	KCl (98%, v/v)	1920	[117]
Lipase				
Pseudomonas cepacia	Anhydrous pyridine	Sorbitol	3	[118]
Mucor javanicus	Toluene	KCl (98%, v/v)	5	[119]
Pseudomonas stutzeri (lipase TL)	Anhydrous 2-methylbutan-2-ol	17-β-Oestradiol 3-carboxymethyl ether	26	[82]
Humicola lanuginosa	Hexane	KCl (98%, v/v)	46	[120]
Humicola lanuginosa	Hexane	18-Crown-6[c]	40	[74]
Mucor javanicus	Hexane	KCl (98%, v/v)	11	[74]
Lipoprotein lipase (*Pseudomonas* sp.)	Anhydrous pyridine	Sorbitol	8	[116]
Horseradish peroxidase	Acetone (97%, v/v)	o-Hydroxybenzyl alcohol	62	[118]
	Propan-2-ol[b] (99.4%, v/v)	o-Hydroxybenzyl alcohol	65	[118]
	Acetone (97%, v/v)	Urea (8 M)	56	[79]
	Water-saturated hexane	Urea (8 M)	350	[79]
Soybean peroxidase	Acetone (99.5%, v/v)	o-Hydroxybenzyl alcohol	400	[118]
		m-Hydroxybenzyl alcohol	100	[118]
		trans-1,2-Cyclohexanediol	110	[118]
		Guaiacol + poly(ethylene glycol)	450	[118]
		trans-1,2-Cyclohexanediol + poly(ethylene glycol)	800	[118]
Phospholipase D	Chloroform	KCl (98%, v/v)	10	[121]
Penicillin amidase	Hexane	KCl (98%, v/v)	750	[122]
	Acetonitrile	KCl (98%, v/v)	225	[122]

[a] Synonym t-amyl alcohol.
[b] Synonym isopropanol.
[c] A crown ether.

of the phenomenon of pH memory [67]. It is now clear that the enzyme activity in nearly anhydrous media depends (just like it does in conventional aqueous medium) on the ionization state of the active-site residues. Thus the so-called 'pH memory' operates because, upon freeze-drying, side chains of amino acids retain their ionized/un-ionized state. Hence, the freeze-dried powders show enhanced activity as a result of pH tuning. However, "In aqueous solutions, counterions can freely move around in a solution. Because they are not closely associated with opposite charges, their identity does not affect the protonation state of the enzyme. Thus, pH alone governs the protonation state. When a biocatalyst is suspended in a low water organic solvent, the situation is more complex. In this case, counterions are in closer contact with the opposite charges on the enzyme because of the lower dielectric constant of the medium. Thus, protonation of ionizable groups on the enzyme will be controlled by the type and availability of these ions as well as hydrogen ions" [68].

Several other ways of fixing the 'apparent pH' of enzyme powders have been described in the literature.

(a) Organic soluble buffers

Some pairs of acids and their salts are available which are soluble in organic solvents. Blackwood et al. [69] used triphenylacetic acid and its sodium salt to optimize the catalytic performance of subtilisin in pentanone. Similarly, tri-iso-octylamine and its hydrochloride salt were used to enhance the activity of lipase in pentanone [69]. For use in more non-polar solvents, dendritic polybenzyl ether and its sodium salt have been described [70]. A list of such soluble buffers has been provided elsewhere [68].

(b) Solid-state buffers

These pairs remain insoluble in the reaction medium, but exchange H^+/counterions with the protein molecule. Each buffer pair, of course, fixes only one value of the ionization parameter. An example of this approach is the use of

lysine/lysine hydrochloride for control of pH + pCl (= − log [Cl^-]) in the case of subtilisin-catalysed reaction in hexane and toluene [71].

Vakos et al. [72] have exploited 'pH memory' effect to develop selectivity for a chemical modification reaction. It was shown that, when proteins were freeze-dried from aqueous solutions at pH values between 6.0 and 7.0, the reaction with iodomethane in a vacuum was restricted to α-amino groups. Reaction with ^{13}C-labelled iodomethane made tentative identification of N-terminal amino acids by ^{13}C-NMR possible. The approach is especially valuable in checking whether a protein has a free or blocked N-terminus. This innovative idea can be extended to many other situations wherein it may be advantageous to limit chemical modification with not-so-selective reagents to a specific kind of side chain. Identification of active-site residues of enzymes, fine tuning an affinity label reaction and chemical cross-linking with a right 'trade off' between stabilization versus activity loss are some possible potential applications. As many monofunctional and bifunctional chemical modification reagents are soluble in organic solvents, the major constraint is that such reactions have to be carried out with suspensions of freeze-dried powders.

Use of excipients

In an early report, using 98% (w/w) KCl as an excipient during freeze-drying led to a subtilisin preparation with 4000-fold higher k_{cat} in hexane [73]. Later, optimization led to salt activation, resulting in a 20 000-fold increase in k_{cat} for the same reaction [74]. Ru et al. [75] have used the Jones–Dole coefficient to study the effect of salt activation. This parameter evaluates an additive for its influence over water viscosity and thus can be related to preferential hydration of the protein in its presence (see the earlier discussion of preferential exclusion theory under 'Protein stability during freeze–thawing'). Salt activation has also been shown to work for α-chymotrypsin, thermolysin, lipase and penicillin amidase [66]. An important application of salt activation, cited in [66], is the thermolysin-catalysed regioselective acylation of taxol to form the adipic acid derivative, which is 1700 times more soluble in water and thus overcomes the problem of the low solubility of taxol in water in its therapeutic uses. Similarly, salt-activated subtilisin-acylated doxorubicin showed enhanced potency (in efficacy against a breast-cancer cell line) as compared with doxorubicin. In both cases, the inactivated freeze-dried enzyme showed no significant activity.

An important and key work in this regard is from Mattiasson's group [76], since it describes somewhat different results and conclusions. Their experience was that with α-chymotrypsin, "... addition of potassium chloride could not yield any improvement in activity for the preparation lacking buffer species" [76]. Another important observation was that while the specific activity of freeze-dried enzyme increased with increasing amounts of buffer salts, it decreased with higher amounts of sodium phosphate (above 35 mmol/g of protein). The authors noted the earlier finding that "sodium phosphate differs from other salts because of its capacity to associate with water in distinct structures" [76]. The activating effect of sorbitol was also enhanced when buffer salts were present. The effect of these additives on the transesterification activity of immobilized α-chymotrypsin has also been described by these authors. Recently, in our studies with α-chymotrypsin [77] and tannase [78], the effect of salt activation was found to be only marginal. Additionally, data with a wider range of enzymes are needed before any definite conclusions about the effect of some additives, especially KCl (during freeze-drying), can be drawn.

The presence of urea during freeze-drying led to substantially activated subtilisin and peroxidase preparations [79]. The effect of urea may not be restricted to "partial unfolding of the enzyme before lyophilization", as mentioned in [66]; a more likely explanation is that given by the authors themselves: urea, by H-bonding, may reduce dehydration-induced structural changes. This is confirmed by the fact that no such effect was observed with another denaturant, namely guanidinium chloride [79]. These authors have provided valuable data on the comparative efficacy of salt activation and urea activation. The activation of subtilisin in hexane and tetrahydrofuran was lower for urea than for KCl (126-fold versus 2103-fold in hexane; 45-fold versus 122-fold in tetrahydrofuran), but higher for urea in acetone (18-fold versus 12-fold).

'Molecular imprinting' or 'inhibitor-induced activation' is a somewhat different kind of approach wherein the substrate analogue of an enzyme is present during freeze-drying. Russel and Klibanov [80] have shown that the presence of a competitive inhibitor during freeze-drying increased the activity of subtilisin 100-fold. Later, similar results were reported for peroxidases, chloroperoxidases and myoglobin [66]. In the case of lipase from the yeast *Candida antarctica*, enantioselectivity of the enzyme was enhanced by molecular imprinting [81]. Rich et al. [82] have used molecular imprinting with paclitaxel-2′-adipic acid and salt activation to enhance the activity of thermolysin 110-fold, the two activating approaches being found to be additive in nature.

FTIR spectroscopy has shown that the use of a crown ether resulted in subtilisin having a secondary structure in dioxane very similar to that of the native enzyme [66]. Crown ethers as lyoprotectants also protect the enzyme by forming non-covalent complexes through the ε-NH_2 groups

of lysine residues of enzymes [66]. Santos et al. [83] believe that crown ethers act as molecular imprinters. As crown-ether-activated enzymes show enhanced activity in polar solvents, whereas salt-activated enzymes show increased activity in non-polar solvents, it is possible that both additives activate enzymes by different mechanisms. Cyclodextrins, the glucose oligomers with cavities of diameter 0.47–0.83 nm, are known to stabilize proteins in aqueous solutions [84]. Freeze-drying of subtilisin along with methyl-β-cyclodextrin increased the transesterification activity of the enzyme powder 164-fold in tetrahydrofuran [85]. The effect of cyclodextrin was seen only in hydrophilic solvents, but not in hydrophobic ones like toluene and octane. Additionally, this chiral additive enhanced the enantioselectivity of subtilisin and *Candida rugosa* lipase. Interestingly, the enantioselectivity of lipase was opposite to that of subtilisin.

Alternatives to the use of freeze-dried enzyme powders

Over the years, workers have generally switched over to using enzymes in forms other than simple freeze-dried powders. These are reported to give better catalytic activities in nearly anhydrous media.

Immobilization Both adsorption and covalent coupling [86,87] have been tried. Sol–gel methods have also been reported [88].

Cross-linked enzyme crystals [89] Altus Biologics, Inc. (Cambridge, MA, U.S.A.) has a patented technology for creating CLECs™ (cross-linked enzyme crystals). These are essentially microcrystals of enzymes which have been cross-linked. These are reported to be extremely robust preparations and show high stability at high temperatures as well as in organic solvents.

Soluble enzymes [90,91] It was found as early as the 1980s that enzymes coupled to poly(ethylene glycol) become soluble in nearly anhydrous solvents. Otamari et al. [92] showed that some complexes of enzymes with polymers are also soluble in organic solvents.

Surfactant-modified enzymes Modification of enzymes, especially lipases, with surfactants gives a preparation that solubilizes well, disperses evenly in organic solvents and gives higher inter-esterification activity with 1,3-positional specificity. The lipase from *Pseudomonas* sp. was modified with a surfactant solution of sorbitan monostearate [93] to hydrolyse palm oil most efficiently. Isono et al. [94] have described the esterification activity of sorbitan monostearate-modified lipase from *Rhizopus japonicus* for wax-ester synthesis.

Enzyme precipitates This avoids the step of freeze-drying. The enzyme is precipitated from its solution in aqueous buffer by adding an organic solvent [95]. Various solvents, such as 2-methylpropan-2-ol (t-butyl alcohol), propan-2-ol (isopropanol), ethanol or 2-methylbutan-2-ol (t-amyl alcohol) have been recommended [96]. The excess water is removed by repeated suspension and precipitation of the enzyme solution with the co-solvent. It should be added that precipitation with organic solvents has been a classical practice in enzymology for the concentration and fractionation of enzymes. Further details on this approach may be found elsewhere [97–99]. On the basis of this approach, Moore et al. have described propanol-rinsed enzyme preparations ('PREPs') [100].

Post-freeze-drying storage stability

Constantino et al. [101] have provided examples of how freeze-dried proteins can become denatured in the presence of moisture. BSA, freeze-dried from pH 7.3 and stored at 37 °C, with a water content of approx. 40 g/100 g of protein, underwent aggregation (resulting in diminished solubility). Aggregation was via thiol–disulphide interchange. Similar results were obtained for recombinant human albumin. In both cases, freeze-drying from acidic solutions (which ensures that –SH groups are not ionized) prevented aggregation (and the resultant solubility problem). Previously, Constantino et al. [102] reported seven excipients which could prevent moisture-induced aggregation and demonstrated that their stabilizing potency could be correlated with their water-sorbing power. In the case of freeze-dried human insulin at 50 °C and 96% relative humidity, aggregation via β-elimination occurred, followed by thiol-catalysed disulphide exchange. Again, lowering the pH of the solution before freeze-drying reduced both β-elimination and subsequent thiol–disulphide exchange. The third example discussed is that of tetanus toxoid, which illustrates yet another mechanism of deterioration of a freeze-dried protein during storage. Tetanus toxoid formed aggregates, caused by covalent non-disulphide bonds. These bonds were found to be between Schiff-base intermediates (generated from formaldehyde molecules present in the toxoid preparation) and the side chains of lysine, tyrosine and histidine residues in the toxoid molecule. The preventive strategy was succinylation of toxoid or reduction of 'labile formaldehyde linkages' with cyanoborohydride. However, in their previous work [103], the authors reported that freeze-drying produced a reversible reduction in α-helix content (with concomitant increase in β-sheet structure). Thus, probably, freeze-drying *per se* caused only reversible changes in the secondary structure, and aggregation via non-disulphide covalent bonds

was a subsequent phenomenon, i.e. during storage and in the presence of moisture.

Arakawa et al. [2] have also discussed some examples concerning storage stability. In interleukin-2, the non-essential residue Cys^{125} was changed to alanine or serine and led to enhanced storage stability by preventing aggregation. Similarly, in the case of fibroblast growth factor, mutation of two solvent-exposed cysteine residues improved the shelf-life of the freeze-dried protein. Free cysteine residues (e.g. in granulocyte colony-simulating factor), if buried and unavailable to react with other cysteine residues, do not cause a problem during storage. In general, inactivation mechanisms at this stage are identical with those which have been known to operate in solution. Hence all the strategies that work in stabilizing proteins in solution should work at the storage stage, which is, after all, dominated by moisture-triggered structural changes.

Finally, it is necessary to recall the relevance of T_g to storage stability, which has been briefly referred to above (see the section 'Basic process'). It is believed that proteins are more stable when stored at temperatures below their T_g [9]. It has, however, been pointed out that, although storage below T_g is advantageous, it does not guarantee stability. This is because molecular mobility still exists below this temperature [8]. It is also necessary to realize that even a trace of moisture may bring the T_g to storage temperature and cause deactivation. It has been suggested that, as a rule of thumb, the dried products should be stored at least 50 °C below the T_g [8]. This, however, may quite often be impractical.

Freeze-drying: miscellaneous issues

Use of freeze-drying is not restricted to proteins alone. It has become a widely used process in many areas of chemistry, pharmacy and biology. Brown et al. [104] have examined the stability of retinal, α-tocopherol, *trans*-lyopene and *trans*-β-carotene in freeze-dried serum. Chongprasert et al. [105] showed that the crystal forms of the drug pentamidine isethionate present after freeze-drying depend on the initial solution concentration and the thermal history of the sample before freezing. It is pointed out that "seemingly subtle differences in processing conditions can have a significant impact on the critical quality attributes of freeze-dried products". Earlier, van Winden et al. [106] had looked at the stability of liposomes during freeze-drying and concluded that slow freezing resulted in a marked retention of encapsulated fluorescent molecule after freeze-drying and rehydration, as compared with rapid freezing. Thus quick freezing affects the integrity/stability of liposomes.

The freeze-dried plasma standard for calibrating the fibrinogen assay was introduced in 1992 by The National Institute for Biological Standards and Control (NIBSC, South Mimms, Potters Bar, Herts., U.K.). A recent study examined the influence of freeze-drying on the clotting properties of fibrinogen in plasma [107]. The authors concluded, "... findings support the use of a freeze-dried international plasma calibrator, also in fibrinogen assays based on measurements of clotting rate. In epidemiological studies, however, when comparing minor differences in fibrinogen concentrations, the influence of freeze-drying on the clotting rate of calibrations plasma should be taken into account" [107]. In general, there is obviously a need to be cautious when using freeze-dried proteins in analytical methods. Haddeland et al. [108] have described an interesting example of how freeze-drying can sometimes give rise to a novel biological property. It was found that freeze-dried fibrinogen stimulated tissue-type-plasminogen-activator-catalysed plasminogen activator. The conformational changes during freeze-drying presumably exposed 'stimulatory sites' which were inaccessible in the native fibrinogen molecule.

Conclusions

DePaz et al. [109] pointed out that, in some cases, storage stability of some peptides/protein (in solid/powder form) is even less than in aqueous solutions. Thus a greater understanding of the freeze-drying process may result in a rational, and hopefully more stable, formulation for enzymes and pharmaceuticals. If freeze-drying damages the protein conformation (and catalytic activity), why did it escape the notice of biochemists? In other words, why was freeze-drying perceived as a gentle way of concentrating proteins? The answer to this question lies in the fact that enzymology (and our expectations from enzymes!) have undergone subtle changes over the years. Earlier, biochemists were using proteins/enzymes only in aqueous buffers. As structural changes during freeze-drying are mostly reversible during rehydration, the deleterious effects of freeze-drying escaped notice. Today, enzymes are used in nearly anhydrous organic solvents [4], reverse micelles [110], solvent-free systems [111] and the frozen state [112]. Also, production processes are expected to result in preparations with reproducible biological activity, especially in the case of pharmaceutical proteins and protein-based vaccines. Driven by a 'balance sheet', it is also desirable to have as highly active a catalyst as possible during process optimization. Hence the current focus on this overlooked process, which was used rather empirically. It has been pointed out that one of the limiting factors in gene therapy is the instability of viruses [86]. For

example, retroviruses get inactivated during freeze-drying rather easily. It is likely that our understanding of freeze-drying can ultimately be extended to these more complex nucleoproteins. This optimistic note is not without basis. Trehalose, which protects protein structure during thermal stress and drying, does have a protective function on more complex structures. "Trehalose is widely found in nature among diverse species which are capable of withstanding conditions where they are exposed to conditions of low water activity and/or thermal stress, e.g. bacteria, yeast, mushroom, nematodes, shrimp and desert plants" [113]. Thus freeze-drying (lyophilization) may turn out to be a 'Cinderella' among the techniques which enzymologists have been using over the years. The fascination with this technique, although of relatively recent origin, is already paying rich dividends in many process designs [114,115].

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